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DNA damage in a liver tissue of metal exposed *Clethrionomys glareolus*

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HIGHLIGHTS

- Aim of the study was assessment of DNA damage (AP sites) in tissues of *C. glareolus*.
- ANOVA showed no difference in number of AP sites between single populations.
- T-test showed significant difference between unpolluted and polluted populations.
- There were relationships between number of AP sites and metal concentrations.

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ABSTRACT

It is widely known that some toxic agents may act on DNA strand resulting in its damages. One of the possible impairments is formation of abasic sites in DNA. The aim of this study was to indicate a presence of these DNA sites in the liver tissue of bank voles inhabiting the vicinity of zinc/lead smelters. Samples that were used originated from animals collected from unpolluted (Niepołomice, Teleśnica Oszwarowa, Mikołajki) and polluted (Miasteczko Śląskie, Katowice, Olkusz) populations. They significantly differed in terms of tissue lead concentrations in the kidney and liver. The means of detected AP sites per 10^5 bp ranged between 3.39 (Teleśnica Oszwarowa) to 5.13 (Miasteczko Śląskie). Statistical analysis (ANOVA) showed no difference in terms of number of the AP sites between single populations. However, t-test showed significant difference between the unpolluted and polluted populations. Factorial ANOVA indicated that sex is not a factor influencing the number of AP sites. The analyses revealed statistically significant relationships between the number of AP sites and Cu concentrations in the liver, and also Pb and Cd concentrations in the kidney.

1. Introduction

Metal pollution is a serious problem for animal and human populations, especially since human activity causes substantial alterations in the environment and increases emission of different toxicants. Various aspects of metals' (such as cadmium, lead or zinc) toxicity has been studied so far (Świergosz-Kowalewska et al., 2006; Vanparys et al., 2008; Wang and Fowler, 2008) including direct and indirect metal toxicity at different levels of biological organization, from cell to population.

It is widely known that some toxic agents may act on DNA strand resulting in damage of DNA molecule (Kumari et al., 2008). A specific type of damage that can be detected may thus serve as a biomarker of exposure (Shugart, 2000). This assessment is implemented in many studies, although there is no universal biomarker for inferring DNA damage. This kind of adverse effect may have different origins and reflect different type of DNA strand breakdown.

Oxidative stress is one of the most extensively studied factor acting on DNA strand (Koivula and Eeva, 2010). Redox-active (iron, copper, chromium) and redox-inactive metals (lead, cadmium, and mercury) may considerably increase production of reactive oxygen species. When defence capacity of cell is exceeded, oxidative stress is strengthened in the cell. Oxidative stress is responsible for damage to DNA, but also for damage to lipids and proteins. Increasing number of studies addressing this issue shows that oxidative stress may be an important aspect of metal toxicity (Ercal et al., 2001).

Up till now, different markers of oxidative DNA damage have been used (Nikitaki et al., 2015; Valavanidis et al., 2006), like comet assay (Collins, 2004; Azqueta and Collins, 2013) or detection of oxo^{8}dG (Shigenaga et al., 1994). One of the methods is assessment

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of an abasic sites number (Nakamura and Swenberg, 1999; Kubo et al., 1992). Apurinic/apyrimidinic sites (AP sites) are DNA lesion formed after the cleavage of N-glycosyl bond by DNA glycosylase, as well as by the spontaneous process called depurination. Under normal conditions this process is balanced by repairing mechanism involving the AP endonucleases. It has been shown that imbalance in the AP sites repair system may cause carcinogenesis (Nakamura and Swenberg, 1999). The AP sites can also negatively influence DNA replication.

The aim of this research was to study a number of the abasic sites in DNA strands isolated from the liver tissues of animals inhabiting surroundings of zinc/lead smelters and also unpolluted sites. Heavy metal concentrations in the liver and kidney tissues of bank vole, determined in the previous study, showed significant differences between the clean and polluted populations (Mikowska et al., 2014) suggesting that some effects at a genetic level could appear. To confirm our hypothesis, the number of abasic sites were measured with colorimetric method described by Kubo et al. (1992). We chose the liver, because as a metabolically active organ (where accumulation and also detoxification processes of various toxicants occur) it can be a good representative tissue - the DNA damages will be more severe than in other tissues.

2. Material and methods

2.1. Study sites and animal collection

The study was performed on six populations of wild bank voles (*Clethrionomys glareolus*) from different study sites. Bank voles have been studied in previous investigation and the same tissue material was used here (Mikowska et al., 2014). The animals were trapped at three sites located in Southern Poland, close to zinc/lead smelters: (1) Miasteczko Śląskie, (2) Katowice, (3) Olkusz, and at three unpolluted sites located in Southern and Northern Poland: (4) Niepołomice, (5) Teleśnica Oszwarowa, (6) Mikołajki. Site classification, as unpolluted and polluted, was based on the information concerning the industrial operations in these areas, which had a long history of lead/zinc mining and smelting. Detailed information on location of the sites, as well as references to previous research are presented in paper by Mikowska et al. (2014).

The animals were live trapped during late summer and autumn in 2009 and transported to a laboratory, where they were dissected. The liver tissues were frozen in −80 °C. DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to incorporated protocol. Following this procedure, DNA concentration was measured using Nanodrop ND-1000 spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Mean value of three measurements per sample was used for subsequent procedures to ensure high accuracy. Then, an assay for measuring the number of abasic sites (DNA Damage Quantification Colorimetric Kit, BioVision) was performed according to vendor's protocol with the amount of 0.1 μg of DNA. The number of AP sites per 10^5 bp was calculated based on standard curve in a range from 0 to 40 AP sites/10^5 bp. Pilot studies showed that our samples would fit this range.

2.2. Statistical analysis

The basic data obtained in the study were transformed using natural logarithm to fit normal distribution. Factorial ANOVA (factors: sex, population site) was performed to check for possible effect of sex on the number of AP sites in the six populations. Because sex was not a significant factor, ANOVA test was used to check differences in the number of AP sites/10^5 bp between individual populations and additionally t-test was applied to test differences between the polluted (no. 1–3) and unpolluted (no. 4–6) populations. Regression analyses were used for relationship between lead, cadmium, zinc, iron and copper (mg metal/kg dry weight of tissue) accumulated in the liver and kidney (except copper) and the number of AP sites.

3. Results

The means of detected AP sites per 10^5 bp ranged between 3.39 (Teleśnica Oszwarowa – unpolluted site) to 5.13 (Miasteczko Śląskie – polluted site) (Table 1). Statistical analysis (ANOVA) showed no difference in terms of the number of AP sites between individual populations. However, the t-test showed a significant difference between unpolluted and polluted populations (Fig. 1). Regression analysis revealed statistically significant relationship between number of AP sites and Cu concentration in the liver, and additionally between AP sites and Pb or Cd concentration in the kidney (Fig. 2).

4. Discussion

DNA biomarkers can help to explain how metal exposure affected focal population in the past (such as bottleneck effect, genetic drift, etc.) and can implicate contemporary population condition (genetic biodiversity). However, it is also very important to know if populations inhabiting contaminated sites may suffer from changes at genome level and if this changes may be a result of metal contaminants. Some metals and metalloids (Cd, Cu and Fe) are classified as elements potentially carcinogenic (Bal and Kasprzak, 2002). One of the scientists' assumption is that oxidative stress is caused by those metals and that the complex compounds of those metals may catalyze redox reactions resulting in DNA strand oxidation (bases modification, base adducts creation, DNA breaking in phospho-sugar backbone of DNA and abasic sites production). One of the factors that can decrease antioxidant capacity is cadmium, as showed by Kafel et al. (2012). The authors showed that animals exposed to contaminated diet (44 mg Cd/kg dry mass of diet) during one generation, had higher total antioxidant capacity in comparison to the animals from multigenerations treatment. Of course, natural repairing processes of DNA damage evolved. One of these processes, involving correction of different types of DNA damages, is base excision repair (BER) (Fortini, 2003;

Table 1
Average number of AP sites per 10^5 bp detected in studied populations (SE - standard error).

Site	Average AP sites per 10^5 bp	±SE	Number of individuals	Number of males	Number of females
Katowice[a]	4.83	0.60	9	5	4
Miasteczko Śląskie[a]	5.13	0.60	9	6	3
Mikołajki	4.50	0.60	9	4	5
Niepołomice	3.89	0.60	9	4	5
Olkusz[a]	5.00	0.64	8	4	4
Teleśnica Oszwarowa	3.39	0.60	9	4	5

[a] Populations classified as polluted.

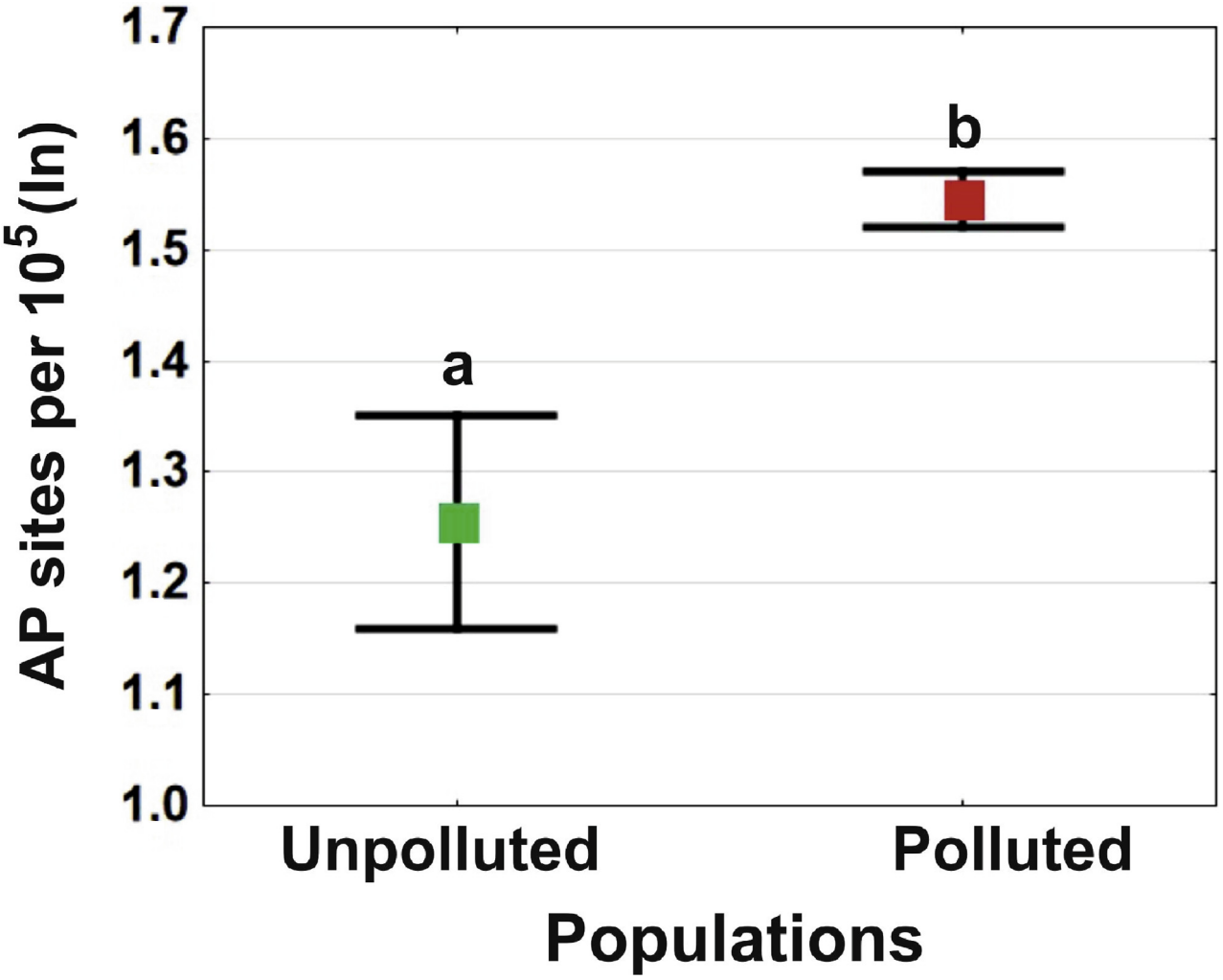

Fig. 1. Statistical difference (a, b; $p < 0.05$) between two groups of populations in the number of AP sites per 10^5 bp (ln). Data transformed using natural logarithm (ln); mean ± standard error.

Krokan and Bjørås, 2013; Hedge et al., 2008; Srivastava et al., 1998; Hill et al., 2001; Kafel et al., 2012). This process consists of five reactions: (1) removal of a base; (2) incision of the abasic site; (3) processing of the generated termini at the strand break; (4) synthesis of DNA and (5) ligation. Occurrence of the abasic sites can influence partial and overall structure of DNA (Dahlmann et al., 2009) and can be caused by DNA alkylation or oxidation. The abasic sites can also occur during repairing damaged nucleotides. In conditions of no stress, cell has a great capacity for such a repair.

We hypothesized, that bank voles from populations inhabiting polluted site could be under stress conditions, which may result in DNA changes. Our study showed that pollution affects DNA, expressed as higher mean number of AP sites in DNA strand of animals from polluted populations than clean ones (Fig. 1). However, overall effect is not strong, when we compare values for both types of populations, the average numbers of AP sites per 10^5 bp ranged from 3.39 to 5.13 (Table 1). To test effects on individual level, relationship analyses between different metals concentration in the liver and kidney tissues and the number of AP sites in the liver were performed. Significant effect was found in case of Pb and Cd in the kidney (Fig. 2) and copper in the liver. Metal concentrations (especially Pb and Cd) found in the studied bank vole tissues (data from Mikowska et al., 2014) were rather low – lower in the liver than in the kidney suggesting high rate of metal elimination through this organ. Thus, kidney metal concentration could better reflect the degree of animal exposure to environmental pollutants and better correlate with DNA changes in the liver, which could cumulate over lifetime.

The method we used in the study employs ARPs (Aldehyde Reactive Probes). To our knowledge, there is not much research using such an approach, and thus, there is not much data to compare with our results. One of the known studies has been done by Aboul-Ela et al. (2011), who assessed biomarkers of oxidative stress (also number of AP sites) in two populations of *Mugil cephalus*. They showed higher concentration of Cu and Fe in the liver tissues from polluted site when compared to clean area and significantly greater number of AP sites in the tissues from animals in contaminated site comparing to clean area (Fe: 407 ± 38 vs. 216 ± 21 μg/g wet wt; Cu: 54 ± 6 vs. 17.7 ± 4 μg/g wet wt). Our results for Fe in the liver ranged from 494.3 ± 73.8 mg/kg dry weight (Katowice) to 924.5 ± 61.2 mg/kg dry weight (Mikołajki), for Cu from 13.1 ± 1.0 mg/kg dry weigh (Teleśnica Oszwarowa) to 18.9 ± 1.0 mg/kg dry weight (Niepołomice). This suggest substantially higher levels of these metals in our samples, as loss of water during oven drying process can be up to 75% in case of frozen tissues (Adrian and Stevens, 1979). The other biomarkers studied in the paper by Aboul-Ela et al. (2011) (MSA concentration and catalase enzyme activity) were also significantly higher in

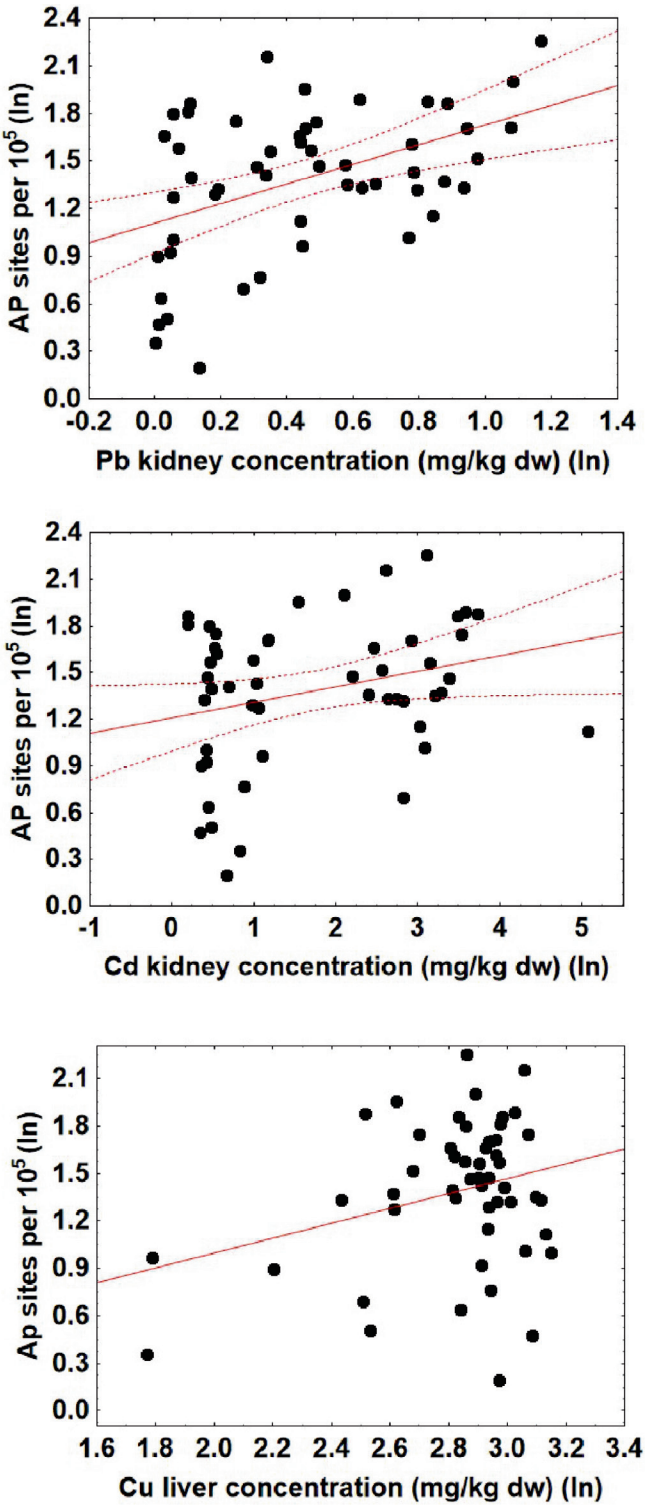

Fig. 2. Relationship between metals concentrations in the tissues (mg/kg dry weight) and the number of AP sites per 10^5 bp sites. Data transformed using natural logarithm (ln); p-value < 0,05.

contaminated site than in the clean site. They found significant differences between sites for both the liver and gills. However, AP values for gills were rather low: 1.99 (polluted area) and 0.19 (clean area), suggesting low negative pressure of pollution on this tissue. If it comes to the liver, AP levels found by Aboul-Ela et al. (2011) were much higher than found in ours study on the bank voles reaching value 13.98 for polluted area and much lower (0.37) than the lowest values found for the bank voles. In our study, the lowest number of AP sites was 1.21 and it was noted for individual from relatively unpolluted Niepołomice population. Ranges showed by Aboul-Ela et al. (2011), suggest that there were individuals with almost no AP sites detected.

Our result, with its range from 3.39 to 5.13 AP sites, do not show such discord between polluted and unpolluted sites. We may hypothesize that this level is moderate, present in the environment affected by anthropogenic impact. This could explain the fact, why Mikołajki population (classified as unpolluted) is characterised by comparable level of the AP sites to Katowice (polluted).

Number of studies concerning DNA damage have employed other methods than assessment of abasic sites: comet assay (Collins, 2004; Azqueta and Collins, 2013) or detection of oxo^{8}dG (Shigenaga et al., 1994). One of the most popular and most extensively used method of measuring DNA damage is the comet assay. Danadevi et al. (2003) performed their DNA damage assessment in leucocytes of workers exposed to lead. The percentage of damaged cells was greater in an exposed group (44.58%) when compared to control group (21.14%). However, the difference in the level of damage among the exposed individuals does not reflect the difference between lead concentration in blood - 248.3 μg/L and 27.49 μg/L respectively. The comet assay was also used in ecotoxicological research by Pruski and Dixon (2002), who were studying damaging potential of cadmium on DNA of mussel *Mytillus eduli*. They found no genotoxic effect of cadmium in the gill tissues (*in vivo* exposure to $CdCl_2$ - 0.2 mg/L for 4 weeks). Interesting fact was that exposure to low doses of cadmium (*in vitro* exposure to $CdCl_2$ - 0.2 mg/L for 4 days) enhances genotoxicity of H_2O_2. Usually, the negative effect of H_2O_2 was reversible after return to clean medium. The efficient repair mechanisms were then possible thanks to present zinc ions. However, after intoxication with Cd, zinc was displaced from active sites of enzymes, in consequence causing harm to the cells. The method of comet assay is still developing, being a good choice for both *in vivo* and *in vitro* studies in laboratory and under environmental conditions (de Lapuente et al., 2015). This method was also used for rodent studies (Uno et al., 2015). Heuser et al. (2002) used it for rodent biomonitoring (*Ctenomys miniuts)* in the regions exposed to vehicle emission.

Another method was used by Chater et al. (2008), who intoxicated pregnant female rats with cadmium chloride (3 mg/kg body weight) and checked oxidative DNA damage by measuring the level of 8-oxodGuo. Results showed that there was no increase of 8-oxodGuo levels in the livers, but in the kidneys they noted 51% of increase. The above mentioned results did confirm the impact of Cd exposure on DNA damage in cells.

5. Conclusion

As we demonstrated, the AP sites measurement may be a good and sensitive method to estimate effects of metal exposure to DNA, even though the metal intoxication is not high. This statement is supported by our results concerning studied populations: although values of AP sites (thus, the intensity of an impact) were not very high (up to 9.53), we could still see statistical differences between unpolluted and polluted populations. Our experiment was performed in the field, but we are sure that DNA damage method may be also successfully applied under laboratory conditions. We cannot exclude that presence of other contaminants (not measured by us) could contribute to AP sites formation. Such situation is most likely to happen in case of Mikołajki site (classified as unpolluted) where intensity of DNA changes is similar to this obtained for polluted sites.

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